REMARKS

This Response is being filed in connection with the Office Action mailed January 26, 2005. Claims 1 to 30 are pending. Claims 22 to 28 and 30 stand withdrawn from consideration as directed to a non-elected invention. Claims 1 to 7, 12 to 19 and 29 have been canceled herein without prejudice. Applicants maintain the right to prosecute the canceled claims in any related application claiming the benefit of priority of the subject application. New claim 31, which depends from claims 8 through 11, has been added. Accordingly, upon entry of the amendment, claims 8 to 11, 20, 21 and 31 are under consideration.

Regarding the Amendments to the Specification

The specification has been amended to insert trademark symbols, "TM." The amendment was made in order to address the Examiner's request to address this informality. Accordingly, the amendments to the Specification do not introduce new matter and entry thereof is respectfully requested.

Regarding the Claim Amendments

Claims 20 and 21 have been amended to recite that new claim dependency. The amendments reflect the cancellation of claims 1 to 7 and 12 to 19 and, therefore, were made to address an informality. Accordingly, the claim amendments do not introduce new matter and entry thereof is respectfully requested.

Regarding the New Claim

Support for new claim 31 can be found throughout the specification, for example, at page 18, lines 20-21; page 19, lines 9-11; page 22, lines 14-19; page 26, lines 14-17; page 26, line 27, to page 28, line 3; page 28, line 3, to page 29, line 17. Accordingly, new claim 31 does not introduce new matter and entry thereof is respectfully requested.

I. REJECTIONS UNDER 35 U.S.C. §112

The rejection of claims 4 to 7, 14, 15, 17 and 20 under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement is respectfully traversed. Allegedly, deposits of the antibody

producing hybridomas are required in order to satisfy the enablement requirement under 35 U.S.C. §112.

The claims, as filed, are adequately enabled. Nevertheless, solely in order to further prosecution of the application and without acquiescing to the propriety of the rejection, claims 1 to 7, 12 to 19 and 29 have been canceled herein without prejudice. Accordingly, the rejection of these claims is moot.

As to the rejection of claim 20, Applicants submit that deposits of certain antibodies have been made, and the deposit date, name and address of the depository is disclosed in the application. In particular, Applicants respectfully direct the Examiner's attention to page 16, line 20, to page 17, line 1; and page 18, line 20, to page 19, line 8. In view of the foregoing, Applicants believe that the deposit requirements have been satisfied. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph be withdrawn.

The rejection of claims 4 to 7, 14, 15, 17 and 20, under 35 U.S.C. §112, second paragraph, as allegedly indefinite is respectfully traversed. The Examiner indicates that the claims are indefinite due to the use of laboratory designations for the antibodies.

The claims, as filed, are adequately described. Nevertheless, solely in order to further prosecution of the application and without acquiescing to the propriety of the rejection, claims 1 to 7, 12 to 19 and 29 have been canceled herein without prejudice. Accordingly, the rejection of claims 4 to 7, 14, 15 and 17 is moot.

As to claim 20, this claim, as amended, depends from claims 8 through 11. Claims 8 though 11 do not recite any of the laboratory designations F1-102, F5-152, F2-103, F5-157, 72 or F4-465. Thus, because amended claim 20 does not recite any of the foregoing laboratory designations, the grounds for rejection do not apply to amended claim 20. Accordingly, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. §112, second paragraph.

II. <u>REJECTIONS UNDER 35 U.S.C. §102</u>

<u>U.S. Patent No. 5,874,082 (De Boer)</u>

The rejection of claims 1 to 11 and 19 to 21, under 35 U.S.C. §102(e) as allegedly anticipated by U.S. Patent No. 5,874,082 (De Boer) is respectfully traversed. Allegedly, de Boer describe agonistic and antagonistic anti-CD40 antibodies.

Anticipation requires the disclosure in a single prior art reference of each element of the claim under consideration *In re Spada*, 911 F.2d 705 (Fed. Cir. 1990), *In re Bond*, 910 F.2d 831 (Fed. Cir. 1990).

Claims 1 to 7, 12 to 19 and 29 have been canceled herein without prejudice. Accordingly, the rejection of claims 1 to 7 and 19 is moot.

As to claims 8 to 11, 20 and 21, these claims are directed to anti-human CD40 antibodies and functional fragments thereof that "inhibits CD40L (1 ug/ml) mediated tonsillar B cell proliferation in vitro." As disclosed in the specification, Claims 8 to 11 recite varying inhibitory efficiencies, namely from "about 50 to 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.01 ug/ml to 10 ug/ml" (claim 8); about 85 to 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.1 ug/ml to 10 ug/ml" (claim 9); about 80 to 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.01 ug/ml to 10 ug/ml" (claim 10); and about a 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.1 ug/ml to 10 ug/ml" (claim 11). However, deBoer does not describe anti-CD40 antibodies or fragments thereof that inhibit CD40L mediated tonsillar B cell proliferation in vitro at the recited inhibitory efficiencies.

deBoer describe two agonist anti-CD40 antibodies, namely S2C6 and G28.5. S2C6 and G28.5 both stimulate B cell proliferation (column 17, lines 59-61; Figures 5A and 5B; specification, page 66, lines 21-28). Thus, because S2C6 and G28.5 stimulate B cell proliferation, whereas claims 8 to 11, 20 and 21 are directed to anti-human CD40 antibodies and functional fragments thereof that "inhibits CD40L (1 ug/ml) mediated tonsillar B cell proliferation in vitro," neither S2C6 nor G28.5 antibodies are within the scope of claims 8 to 11, 20 and 21.

deBoer describe three antagonist anti-CD40 antibodies, namely 5D12, 3A8 and 3C6. Each of 5D12, 3A8 and 3C6 were unable to costimulate B cell proliferation (see, for example,

column 6, lines 48-49; column 10, lines 59-61; and column 17, lines 56-59), and inhibited B cell proliferation (see, for example, column 18, lines 31-33; and column 19, lines 3-6). The efficiency that each of 5D12, 3A8 and 3C6 inhibit tonsillar B cell proliferation is illustrated in Figures 6 and 7. The data in Figures 6 and 7 indicate that 5D12, 3A8 and 3C6 all have virtually the same tonsillar B cell proliferation inhibitory efficiency. deBoer describe producing a humanized 5D12 Fab and a chimeric 5D12. Both humanized Fab and chimeric 5D12 were shown to have the same binding characteristics as the parent 5D12 (column 32, lines 7-14), indicating that the tonsillar B cell proliferation inhibiting efficiency of humanized Fab and chimeric 5D12 will be substantially the same as the parent 5D12. Thus, all of the antagonist antibodies described by deBoer essentially have the same tonsillar B cell proliferation inhibiting efficiency as 5D12.

The specification discloses tonsillar B cell proliferation inhibition studies with the 5D12 antibody described by deBoer. Applicants respectfully direct the Examiner's attention to Example 6, pages 68 to 70, and Figure 10. In particular, 5D12 antibody showed a maximum of 50% inhibition of tonsillar B cell proliferation at 100 ug/ml (page 70, lines 1-3). In contrast, as discussed above, claims 8 to 11 require that the antibodies and functional fragments thereof have greater inhibitory efficiency of tonsillar B cell proliferation. In particular, claim 8, for example, requires an inhibitory efficiency that leads to about 50 to 95% or greater reduction in tonsillar B cell proliferation when the concentration of the antibody is in the range of 0.01 ug/ml to 10 ug/ml. Thus, because 5D12 lacks the requisite inhibitory efficiency in tonsillar B cell proliferation, and all of the antagonist antibodies described by deBoer have essentially the same tonsillar B cell proliferation inhibiting efficiency as 5D12, none of 5D12, humanized Fab or chimeric 5D12, 3A8 and 3C6 are within the scope of claims 8 to 11, 20 and 21.

In sum, deBoer fails to teach or suggest anti-CD40 antibodies and fragments thereof having the requisite inhibitory efficiency of tonsillar B cell proliferation, as required in claims 8 to 11, 20 and 21. Accordingly, as deBoer fail to teach or suggest each and every element of claims 8 to 11, 20 and 21, the claims are not anticipated by deBoer and Applicants respectfully request that the rejection under 35 U.S.C. §102(e) be withdrawn.

<u>*U.S. Patent Application Publication 2004/0235074 A1 (Siegall et al.)*</u>

The rejection of claims 1 to 11 and 19 to 21, under 35 U.S.C. §102(e) as allegedly anticipated by U.S. Patent Application Publication 2004/0235074 A1 (Siegall *et al.*) is respectfully traversed. Allegedly, Siegall *et al.* describe both agonistic and antagonistic anti-CD40 antibodies.

Claims 1 to 7, 12 to 19 and 29 have been canceled herein without prejudice. Accordingly, the rejection of claims 1 to 7 and 19 is moot.

As to claims 8 to 11, 20 and 21, as discussed above these claims are directed to anti-human CD40 antibodies and functional fragments thereof that "inhibits CD40L (1 ug/ml) mediated tonsillar B cell proliferation in vitro." Claims 8 to 11 recite varying inhibitory efficiencies, namely from "about 50 to 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.01 ug/ml to 10 ug/ml" (claim 8); about 85 to 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.1 ug/ml to 10 ug/ml" (claim 9); about 80 to 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.01 ug/ml to 10 ug/ml" (claim 10); and about a 95% or greater reduction in B cell proliferation when the concentration of the antibody is in the range of 0.1 ug/ml to 10 ug/ml" (claim 11). However, Siegall *et al.* do not describe anti-CD40 antibodies or fragments thereof that inhibit CD40L mediated tonsillar B cell proliferation in vitro at the recited inhibitory efficiencies.

Siegall *et al.* describe four agonist anti-CD40 antibodies, namely 5C3, HuCD40-M2, S2C6 and G28.5. 5C3 increases interaction between CD40 and CD40L (page 1, [0006]). G28.5 and S2C6 stimulate B cell proliferation (page 1, [0009] and [0010]; page 2, [0011]). HuCD40-M2 is reported to inhibit CD40 binding to CD40L (page 1, [0007]. HuCD40-M2 antibody stimulates proliferation of tonsillar B cells (see, for example, page 14, first column, last paragraph and Table 4 of Pound *et al.*, <u>International Immunology</u> 11:11 (1999), a copy of which is submitted herewith as Exhibit A). Thus, because 5C3, M2, S2C6 and G28.5 stimulate B cell proliferation, whereas claims 8 to 11, 20 and 21 are directed to anti-human CD40 antibodies and functional fragments thereof that "inhibits CD40L (1 ug/ml) mediated tonsillar B cell proliferation in vitro," none of the 5C3, M2, S2C6 and G28.5 antibodies are within the scope of claims 8 to 11, 20 and 21.

Siegall *et al.* describe the same antagonist anti-CD40 antibodies as deBoer, namely 5D12, 3A8 and 3C6. As discussed above, 5D12, 3A8 and 3C6 all have virtually the same tonsillar B cell proliferation inhibitory efficiency. As also discussed above, the specification discloses that 5D12 antibody showed a maximum of 50% inhibition of tonsillar B cell proliferation at 100 ug/ml (page 70, lines 1-3), whereas claims 8 to 11 require that the antibodies and functional fragments thereof have greater inhibitory efficiency of tonsillar B cell proliferation. Thus, because 5D12 lacks the requisite inhibitory efficiency, none of 5D12, 3A8 and 3C6 are within the scope of claims 8 to 11, 20 and 21.

In sum, Siegall *et al.* fails to teach or suggest anti-CD40 antibodies and fragments thereof having the requisite inhibitory efficiency of tonsillar B cell proliferation, as required in claims 8 to 11, 20 and 21. Accordingly, as Siegall *et al.* fail to teach or suggest each and every element of claims 8 to 11, 20 and 21, the claims are not anticipated by Siegall *et al.* and Applicants respectfully request that the rejection under 35 U.S.C. §102(e) be withdrawn.

U.S. Patent No. 6,482,411 (Ahuja et al.)

The rejection of claims 3 to 7 and 19 to 21, under 35 U.S.C. §102(b) as allegedly anticipated by U.S. Patent No. 6,482,411 (Ahuja *et al.*) is respectfully traversed. Allegedly, Ahuja *et al.* describe agonistic anti-CD40 antibodies.

Claims 1 to 7, 12 to 19 and 29 have been canceled herein without prejudice. Accordingly, the rejection of claims 3 to 7 and 19 is moot.

As to claims 20 and 21, as discussed above these claims depend from claims 8 through 11, which are directed to anti-human CD40 antibodies and functional fragments thereof that "inhibits CD40L (1 ug/ml) mediated tonsillar B cell proliferation in vitro." Claims 8 to 11 recite varying inhibitory efficiencies in tonsillar B cell proliferation. However, Ahuja *et al.* do not describe anti-CD40 antibodies or fragments thereof that inhibit CD40L mediated tonsillar B cell proliferation in vitro at the recited inhibitory efficiencies.

Ahuja *et al.* appear to describe up to 7 anti-CD40 antibodies, namely G28-5, mAb89, EA-5, 17:40. S2C6, HM40-3, 3/23, which are indicated to be "CD40 agonists," and/or "bind to and activate CD40....thus preventing apoptosis" (column 5, lines 24-35; and column 24, lines 31-42). HM40-3 and 3/23 antibodies are described by Pharmingen (San Diego, CA) and are indicated to have agonist antibody characteristics such as promoting B cell proliferation or expression of

costimulatory molecules (e.g., CD80 and CD86, see Exhibits B and C submitted herewith, copies of technical data sheets for HM40-3 and 3/23, respectively). As discussed above, agonist antibodies stimulate proliferation of tonsillar B cells. Thus, because G28-5, mAb89, EA-5, 17:40. S2C6, HM40-3, 3/23 are agonist antibodies, whereas claims 8 to 11 (from which claims 20 and 21 depend) are directed to anti-human CD40 antibodies and functional fragments thereof that "inhibits CD40L (1 ug/ml) mediated tonsillar B cell proliferation in vitro," none of the G28-5, mAb89, EA-5, 17:40. S2C6, HM40-3, 3/23 antibodies are within the scope of claims 20 and 21.

In sum, Ahuja *et al.* fails to teach or suggest anti-CD40 antibodies and fragments thereof having the activity of claims 8 to 11, from which claims 20 and 21 depend. Accordingly, as Ahuja *et al.* fail to teach or suggest each and every element of claims 20 and 21, the claims are not anticipated by Ahuja *et al.* and Applicants respectfully request that the rejection under 35 U.S.C. §102(b) be withdrawn.

CONCLUSION

In summary, for the reasons set forth herein, Applicants maintain that claims 8 to 11, 20, 21 and 31 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (858) 509-4065.

Please charge any fees associated with the submission of this paper to Deposit Account Number 502212. The Commissioner for Patents is also authorized to credit any over payments to the above-referenced Deposit Account.

Respectfully submitted,
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EXHIBIT A

International Immunology, Vol. 11, No. 1, pp. 11–20

Minimal cross-linking and epitope requirements for CD40-dependent suppression of apoptosis contrast with those for promotion of the cell cycle and homotypic adhesions in human B cells

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Abstract

Eight different CD40 mAb shared with soluble trimeric CD40 ligand (sCD40LT) the capacity to rescue germinal center (GC) B cells from spontaneous apoptosis and to suppress antigen receptor-driven apoptosis in group I Burkitt's lymphoma cells. Three mAb (G28-5, M2 and M3) mimicked sCD40LT in its ability to promote strong homotypic adhesion in resting B cells, whereas others (EA5, BL-OGY/C4 and 5C3) failed to stimulate strong clustering. Binding studies revealed that only those mAb that promoted strong B cell clustering bound at, or near to, the CD40L binding site. While all eight mAb and sCD40LT were capable of synergizing with IL-4 or phorbol ester for promoting DNA synthesis in resting B cells, co-stimulus-independent activation of the cells into cycle through CD40 related directly to the extent of receptor cross-linking. Thus, mAb which bound outside the CD40L binding site synergized with sCD40LT for promoting DNA synthesis; maximal levels of stimulation were achieved by presenting any of the mAb on CD32 transfectants in the absence of sCD40LT or by cross-linking bound sCD40LT with a second antibody. Monomeric sCD40L, which was able to promote rescue of GC B cells from apoptosis, was unable to drive resting B cells into cycle. These studies demonstrate that CD40-dependent rescue of human B cells from apoptosis requires minimal cross-linking and is essentially epitope independent, whereas the requirements for promoting cell cycle progression and homotypic adhesion are more stringent. Possible mechanisms underlying these differences and their physiological significance are discussed.

Introduction

CD40 and its ligand (CD40L) are now established as being central to the development of T-dependent B cell responses. For resting B cells, co-engagement of CD40 and antigen receptor results in a lowering of the threshold for triggering through the latter by two orders of magnitude (1). Resting B cells rapidly form large aggregates on CD40 signalling via both LFA-1-dependent (2) and -independent mechanisms (3). IL-4-dependent CD23 production is dramatically augmented on engaging CD40 (4) and the CD40–CD40L interaction provides a permissive cognate signal for IL-4-driven switching to IgE synthesis in naive B cells (5). The selection process occurring in germinal centers (GC) which results in affinity maturation of

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the immune response to thymus-dependent antigens following hypermutation on Ig V-region genes is CD40 dependent: antigen-rescued centrocytes require cognate interaction with T_h cells containing preformed CD40L for their long-term survival and recruitment into a memory pool (6). Neoplastic phenotypic equivalents of GC B cells, as represented by group I (biopsy-like) Burkitt's lymphoma (BL) cell lines, can be effectively rescued from activation-induced programmed death on engaging cell surface CD40 (7).

The diverse functional outcome to CD40 ligation on the various subpopulations may reflect a differential coupling to intracellular signal transduction pathways during B cell development: we have termed this process 'CD40 receptor rewiring' (8). Thus, for example, whereas there is little tyrosine kinase activity stimulated in resting B cells on engaging CD40, extensive phosphorylation of multiple substrates on tyrosine residues has been reported following CD40-dependent stimulation of GC B cells (9). The ultimate response engendered on CD40 ligation may also depend on the degree of receptor cross-linking. Thus, while for both resting and GC B cells ligand in soluble form (either as a mAb or as trimeric CD40L) can promote certain phenotypic changes without significantly influencing proliferation, the multivalent presentation of ligand on a cell surface membrane can drive or maintain active cell cycle in both populations (10). Finally, to account for its multifunctional role, there is the consideration of possible multiple ligands for CD40 as has been described for the nerve growth factor receptor (NGFR) (11) and the tumour necrosis factor receptor (TNFR) (12), which are members of the same receptor family. Comparative functional studies with limited numbers of mAb to CD40 have indicated that different outcomes can be engendered depending upon epitope specificity of binding (13,14). Evidence for a second CD40L has been reported although formal identification through its cloning and sequencing is still awaited (15).

The present study examines critically the extracellular signalling requirements for promoting the diverse phenotypic changes which are seen to occur in resting B cells, GC B cells and neoplastic phenotypic equivalents of the latter, on ligating CD40. Detailed analysis with eight different mAb to CD40 and with CD40L itself reveals that suppression of apoptosis in GC B cells and the promotion of homotypic adhesions or entry into the cell cycle of resting B cells have markedly distinct requirements. Possible implications of these findings to B cell physiology are discussed.

Methods

Reagents

Mouse monoclonal CD40 antibodies BL-OGY/C4, EA5, 5C3, S2C6, G28-5, HB14, M2 and M3 were from the panel submitted to the Fifth International Workshop on Human Leukocyte Typing (16,17). They were IgG1 isotype with the exception of BL-OGY/C4 (IgM). BU1 (mouse IgG2a anti-μ mAb) and BU25 (mouse IgG1 anti-MHC class II) were produced in the Department of Immunology, Birmingham University. Mouse IgG1 anti-CD40L mAb, M79 and M91 were produced by Immunex Corp. (18). Rabbit anti-human IgM coupled to Sepharose beads (BioRad, Richmond, CA) was employed in B cell proliferation experiments.

Murine CD40LT, human CD40LT and human monomeric CD40L were produced as described in Fanslow *et al.* (19), and the human CD40–IgGFc fusion protein (CD40–Fc) as in Fanslow *et al.* (20).

Cells and cell culture

Human tonsillar GC B cell and resting B cell fractions were isolated as previously described (21). Resting tonsillar B cell fractions were >95% $CD19^+$, >70% IgD^+ and ≤2% $CD3^+$, and GC B cell fractions were >93% $CD19^+$, >76% $CD38^+/IgD^-$, >64% $CD77^+$ and ≤3% $CD3^+$ as defined by flow cytometry on a Becton Dickinson FACScan (Becton Dickinson, Cowley, UK) using FITC- and phycoerythrin (PE)-conjugated mAb (Becton Dickinson and Dako, High Wycombe, UK).

Human peripheral blood mononuclear cells (PBMC) were purified from the blood of healthy donors by centrifugation over Histopaque (Sigma, St Louis, MO). B cells were isolated from PBMC by depletion of cells rosetting with aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC) and treatment of remaining cells with B cell Lymphokwik (One Lambda, Los Angeles, CA) for 1 h at 37°C to lyse contaminating non-B cells. The resulting B cell population was >98% $CD20^+$ with no detectable $CD3^+$ T cells as detected by flow cytometry. T cells were purified by recovery of cells rosetting with AET-treated SRBC, lysis of SRBC, and removal of residual B cells and monocytes by plastic adherence.

Cells were cultured at 37°C in a humidified incubator in 5% CO_2/95% air. Culture medium (CM) was RPMI 1640 containing penicillin (100 IU/ml)/streptomycin (100 μg/ml), 2 mM glutamine (Gibco), and 10% (v/v) FCS (Advanced Protein Products, Dudley, West Midlands, UK). B cells ($0.4–2\times10^6$/ml) were cultured in triplicate wells of flat-bottom 96-well microtitre plates (Becton Dickinson Labware, Oxford, UK) in a total volume of 100 or 200 μl/well. The Epstein–Barr-negative group I BL cell line, L3055, was maintained in CM containing prescreened FCS. Mouse L cells transfected with the gene for human CD32 (CD32-L cells) were obtained from DNAX Research Institute (Palo Alto, CA). CD32-L cells were cultured in HAT selection medium consisting of CM containing hypoxanthine (0.1 mM), aminopterin (0.4 μM) and thymidine (16 μM) (Sigma). The adherent CD32-L cells were recovered using 0.02% (w/v) EDTA in PBS (pH 7.0) and resuspended in CM. They were γ irradiated with a dose of 20,000 rad before addition to B cell cultures at a ratio of (B cells:L cells) 10:1.

Competitive binding studies

Inhibition of binding of soluble CD40 to CD40L on T cells. Peripheral blood T cells were cultured with phorbol myristate acetate (PMA; Sigma; 10 ng/ml) and ionomycin (500 ng/ml) (Calbiochem) for 18 h in order to stimulate surface expression of CD40L. Biotinylated CD40–Fc (1 μg/ml) was pre-incubated with CD40 mAb (10 μg/ml) or control mouse monoclonal IgG for 30 min and then incubated with the activated T cells for 30 min. Bound CD40 was revealed by development with streptavidin–PE (Becton Dickinson Mountain View, CA). Background mean fluorescence intensity (MFI) was determined using biotinylated IL-4 receptor–Fc as control Fc protein and

streptavidin–PE. All binding reactions were performed at 4°C in the presence of 0.02% w/v sodium azide.

Inhibition of binding of CD40 mAb S2C6 to CD40 on B cells

Resting tonsillar B cells were incubated with CD40 mAb (10 μg/ml) or MHC class II mAb BU25 (10 μg/ml) for 40 min and then washed with PBS containing 0.1% sodium azide (PBS/azide). Cells were then incubated with biotinylated S2C6 (100 μg/ml) and then washed in PBS/azide. Binding of S2C6 was visualized by incubation of cells with FITC-conjugated streptavidin (Sigma) and, after further washing, quantified by flow cytometry. All incubations were performed for 40 min on melting ice. The percentage binding inhibition was calculated as for CD40–Fc binding to T cells except that background was determined from the MFI of cells incubated without conjugated S2C6.

Surface plasmon resonance (SPR)

SPR studies of CD40–CD40L–CD40 mAb interactions were performed using a BIAcore biosensor (Pharmacia, Piscataway, NJ). All experiments were performed using an indirect immobilization protocol as described in detail in Arend *et al.* (22). Biosensor chips were coupled with a high avidity goat anti-human IgG1 antibody (GaHIg) (Jackson, Baltimore, MD) using the standard amine-coupling kit according to the manufacturer's recommendation. Briefly, chips were activated with a 6 min pulse of *N*-hydroxy succinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride at 3 ml/min followed by a 6 min pulse of antibody (50 μg/ml) at 3 ml/min in 10 mM sodium acetate, pH 5.0. Unreacted coupling reagent was blocked with a 6 min pulse of ethanolamine at 3 ml/min. The antibody-coated chips were coated with CD40–Fc fusion protein (35 μg/ml) using a 13 min pulse at the same flow rate. This led to immobilization of ~500 pg/mm^2 of CD40–Fc with an essentially zero off rate. The anti-human IgG antibody was regenerated with a 2 min pulse of 1 M formic acid at 5 ml/min, which removed all the Fc fusion protein while causing a ~2% loss in the Fc binding activity of the antibody. For testing the effect of anti-CD40 antibodies on sCD40LT–CD40 interactions, chips coated with CD40–Fc–GaHIg complexes were incubated with antibodies with a 35 ml injection at 3 ml/min using 5 μg/ml of antibody, with the exception of G28-5 which was used at 2.5 μg/ml. These conditions routinely gave 90% occupancy (mol/mol) of the CD40–Fc by antibody based on a mol. wt of the CD40–Fc of 120,000. Subsequently the binding activity of the CD40–Fc–anti-CD40 complexes was tested by incubating the chips with murine sCD40LT at 1.2 μg/ml using a 35 ml injection at 3 ml/min. All data were corrected for binding of reagents to chips coated with GaHIg but no CD40–Fc, by substituting buffer only (20 mM HEPES, 0.15 M NaCl, pH 7.4) for the CD40–Fc fusion protein pulse. For the epitope mapping experiments all conditions were essentially the same except that the flow rate during the antibody injections was 2 ml/min.

All BIAcore data were analyzed by non-linear least squares fitting using the Marquandt–Levenberg algorithm. The data were utilized without transformation as relative units versus time, no weighting was applied. The equations used were of the general form:

$$\text{bound}_t = \Sigma_i\, \text{bound}_{\text{inf}i}[1 - \exp(-k_i t)] \text{ for the association phase}$$

and

$$\text{bound}_t = \Sigma_i\, \text{bound}_{0i}[\exp(-k_i t)] \text{ for the dissociation phase}$$

with the index running from 1 to N. $\text{bound}_{\text{inf}}$ is the amount bound at infinite time, bound_0 is the amount bound at zero time and k is the associated rate constant for the ith component. Data were fit with $N = 1$ then 2 then 3. In general $N = 2$ or 3 sufficed to produce a fit beyond which the sum of squares ceased decreasing, showing that further increasing model complexity was not necessary. These methods are essentially as described elsewhere (23). All analyses were programmed either in MLAB (Civilised Software) or in HiQ (Bimillenium). Data from the BIAcore were exported from the instrument background corrected and formatted prior to analysis using Microsoft Excel software.

DNA synthesis

DNA synthesis was determined by thymidine incorporation. After culture with CD40 mAb or sCD40L for the times specified in Results, cells were pulsed for 16–18 h with [^{3}H]thymidine (Amersham International, Amersham UK; 10 μCi/ml in CM, 50 μl/well) and harvested on a Skatron cell harvester (Helis Bio, Newmarket, UK). Assays were performed in triplicate.

Rescue from apoptosis

Spontaneous apoptosis of GC B cells after 24 h in culture was estimated by enumeration of intact and fragmented cells in Romanowski stained cytocentrifuge preparations (24). Percentage rescue from apoptosis was calculated as: [(% intact cells after culture with test reagent) – (% intact cells in control)/100 – (% intact cells in control)]×100.

L3055 cells were induced to undergo apoptosis by culture with anti-μ mAb BU1 (10 μg/ml) and rescue was determined after 24 h as described for GC B cells.

Homotypic adhesions

The extent of aggregation of resting tonsillar B cells was determined semiquantitatively by phase-contrast microscopy of cultures 48 h after addition of CD40 mAb or sCD40LT.

Results

Competitive binding studies and relative epitope mapping

When CD40 mAb were compared for their ability to inhibit the binding of a soluble bivalent CD40–Fc construct to CD40L expressed on activated T cells, a spectrum of activities was found (Table 1). G28-5, M2 and M3 were effective inhibitors suggesting that the epitopes recognized by these antibodies were within or overlapped with the CD40L binding site. EA5, HB14 and BL-OGY/C4 in turn were partial inhibitors, whereas 5C3 was exceptional in enhancing this interaction, indicating recognition by the latter of an epitope outside of and cooperating with the ligand binding site. Consistent with this, all of the CD40 mAb with the exception of 5C3 were able to block the binding of S2C6 to resting B cells (Table 1). G28-5, M2, S2C6 and EA5 were each found to reciprocally cross-block when their competitive binding to immobilized CD40–Fc was meas-

Table 1. Competition between CD40 mAb and CD40L for binding to CD40

	Inhibition (%)								
	BL-OGY/C4	EA5	5C3	G28-5	S2C6	HB14	M2	M3	Control
CD40L on T cells[a]	25	57 ± 24	−32 ± 30	90 ± 9	82 ± 8	37 ± 15	97 ± 3	96 ± 3	8 ± 3[b]
S2C6[c]	92	89	22	90	84	89	73	78	0[d]

[a]Inhibition of binding of biotinylated CD40–Fc (1 μg/ml) to CD40L expressed by activated T cells after pre-incubation with CD40 mAb (10 μg/ml). Values are means ± SD for three to seven experiments; mean of two experiments for BL-OGY/C4.
[b]Inhibition by monoclonal murine IgG (10 μg/ml).
[c]Inhibition of binding of biotinylated CD40 mAb S2C6 (100 μg/ml) to resting tonsillar B cells by pre-incubation of cells with CD40 mAb (10 μg/ml).
[d]Inhibition by MHC class II mAb, BU25(10 μg/ml).
For further details see Methods.

ured by SPR (Fig. 1). The effect of CD40 mAb on the interaction between CD40 and CD40L was also assessed by measuring the association and dissociation of sCD40LT from immobilized CD40–Fc by SPR. Again G28-5 and M2 were strong inhibitors of this interaction (Table 2). S2C6, EA5 and 5C3 were partial inhibitors however, EA5 and 5C3 also acted to accelerate both the association and dissociation of ligand and receptor (Table 2 and Fig. 2).

All CD40 mAb promote rescue of B cells from apoptosis but only those competing for the CD40L binding site induce homotypic adhesions

Those CD40 mAb which could strongly inhibit CD40L binding mimicked the effect of sCD40LT in inducing homotypic adhesion of resting B cells, whereas those which were poor inhibitors did not promote clustering (Table 3); these differences were seen reproducibly over several experiments with a range of mAb concentrations (up to 5 μg/ml: data not detailed). In contrast, all of the antibodies shared with sCD40LT the ability to rescue both GC B cells and BL cells from apoptosis (Table 3). For GC B cells this was assessed as suppression of their spontaneous apoptosis as judged by morphological criteria; for L3055 BL cells as rescue from the almost complete apoptosis promoted by the anti-IgM mAb BU1 assessed either morphologically or by the ability to restore the DNA synthesis otherwise inhibited as the cells enter growth arrest prior to undergoing apoptosis. Both assays have previously been shown to be robust markers for the detection of apoptosis occurring in normal GC B cells and the L3055 cell line (7,9,10,24,25).

CD40 epitopes can cooperate to stimulate DNA synthesis

All of the CD40 mAb and sCD40LT were effective in stimulating tonsillar resting B cells to low-rate DNA synthesis and acted synergistically with IL-4 or PMA to stimulate high-rate responses (Table 4). Those antibodies which were poor or partial inhibitors of CD40L binding (5C3, EA5, BL-OGY/C4, HB14 and S2C6) synergized with sCD40LT in promoting DNA synthesis in the absence of co-stimulants (Table 5), whereas the strong inhibitors (M2, M3 and G28-5) showed no evidence of this cooperative interaction. Paired combination of the majority of CD40 mAb in the absence of sCD40LT did not result in a synergistic or additive effect; however, stimulation of DNA synthesis by 5C3 was additive with the effect of mAb M2 and M3 (Table 5). The data sets shown in Tables 4 and 5 are representative of three similar experiments.

Evidence of enhanced stimulation occurring through coligation of distinct CD40 epitopes was also evident in peripheral blood B cells. For these cells, EA5 but not M2 was able to synergize with sCD40LT in promoting DNA synthesis (Fig. 3). Co-stimulation of the cells with IgM or IL-4 also revealed functional interaction between S2C6 [which partially blocks CD40L binding (Table 1)] and sCD40LT.

Co-factor-independent stimulation of DNA synthesis requires extensive cross-linking of CD40

When L cells transfected with CD32 were present in the cultures CD40 mAb were able to stimulate high-rate DNA synthesis by tonsillar resting or GC B cells regardless of epitope specificity (Fig. 4B and D). These responses were maximal as indicated by comparison with the effect of combined stimulation with PMA and ionomycin. They could not therefore be further enhanced by sCD40LT as was observed in the absence of CD32 transfectants (Fig. 4A and C). This suggests that co-factor-independent stimulation via CD40 can occur as a result of the extensive cross-linking facilitated by secondary interaction of mAb Fc regions with transfectant-bearing CD32. Similarly, peripheral blood B cells could be stimulated to high-rate DNA synthesis by sCD40LT in the presence of a CD40L mAb which recognized an epitope outside the CD40 binding site (and therefore able to cross-link CD40) but not by a mAb which blocked CD40 binding (Fig. 5). Again, these results are provided as representative data sets from several identical experiments.

Monomeric CD40L can rescue B cells from apoptosis but cannot stimulate DNA synthesis

A monomeric human sCD40L construct was found to be almost as effective as both the trimeric human sCD40LT and the dimeric G28-5 CD40 mAb in rescuing GC B cells from spontaneous apoptosis when used at equivalent concentrations (Fig. 6A). In marked contrast, monomeric sCD40L was unable to stimulate resting B cells into DNA synthesis, whereas the trimeric construct promoted sub-optimal, but still significant, DNA synthesis under otherwise identical conditions (Fig. 6B).

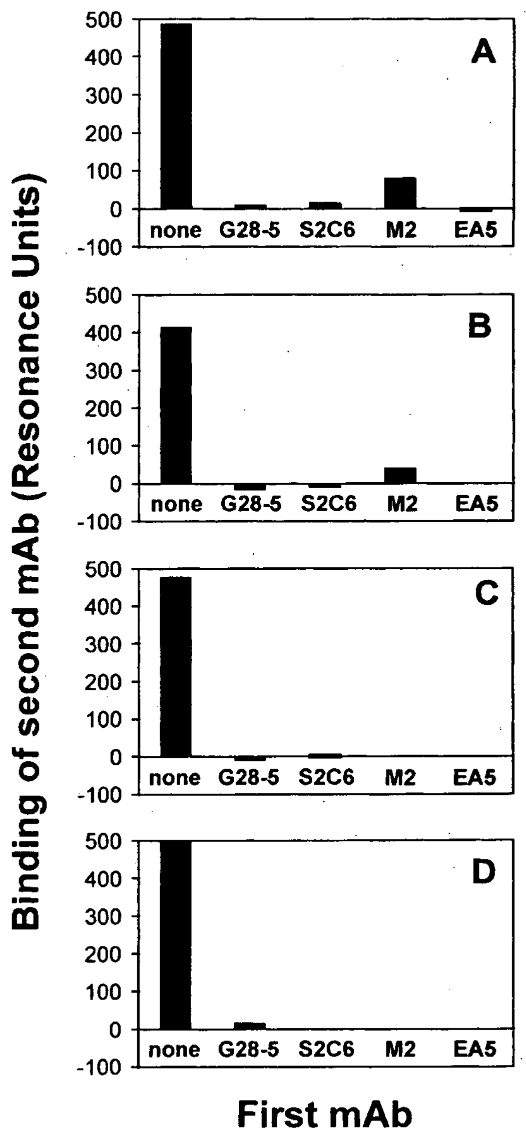

Fig. 1. Competition between CD40 mAb for binding to immobilized CD40–Fc. CD40–Fc was immobilized on a biosensor chip and a CD40 mAb allowed to bind until CD40–Fc was 90% saturated with the antibody. Each panel (A–D) shows the binding of a second CD40 antibody as determined by SPR. (A) EA5, (B) M2, (C) S2C6 and (D) G28-5. Antibodies were employed at a concentration of 5 μg/ml with the exception of G28-5, which was used at 2.5 μg/ml.

Table 2. Effects of CD40 mAb on the kinetics of binding of sCD40L trimer to immobilized CD40–Fc

	G28-5	S2C6	M2	EA5	5C3
Inhibition (%)[a]	75	44	100	49 ± 18[b]	34
Relative on rate[c]	ND	2	ND	12 ± 4[b]	13
Rapid dissociation (%)[d]	ND	10	ND	50 ± 22[b]	44

CD40–Fc was immobilized on a biosensor chip and the binding of sCD40LT in the presence of CD40 mAb was measured by SPR. All antibodies were employed at a concentration of 5 μg/ml and CD40LT at 1.2 μg/ml.

[a]Reduction in the amount of sCD40L bound relative to binding in the absence of mAb.

[b]Mean ± SD for three experiments.

[c]Ratio of the on rate in the presence of CD40 mAb to the on rate in the absence of mAb.

[d]Determined as described in Methods.

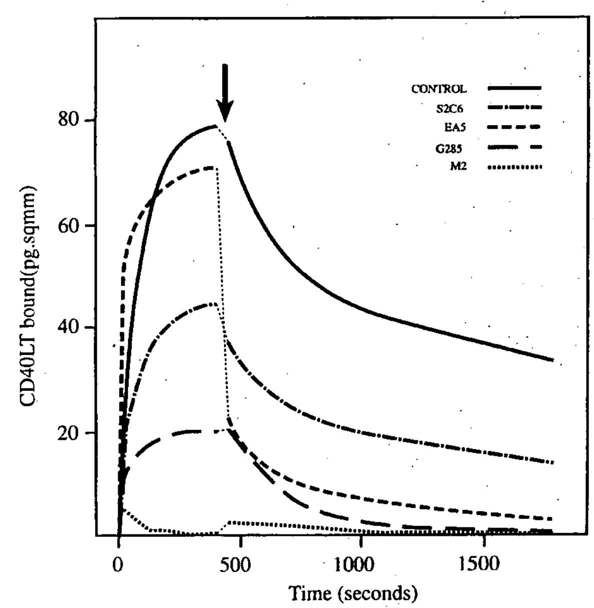

Fig. 2. Effect of CD40 mAb on kinetics of binding of sCD40L trimer to CD40–Fc. CD40–Fc was immobilized on a biosensor chip and the binding of sCD40LT in the presence of CD40 mAb was measured by SPR. Antibodies were employed at a concentration of 5 μg/ml with the exception of G28-5, which was used at 2.5 μg/ml; sCD40LT was used at 1.2 μg/ml. The arrow indicates the point at which CD40LT was replaced with buffer only in the injection.

Discussion

The present study demonstrates that, with respect to extracellular considerations, there are distinct requirements when signalling through CD40 for the induction of homotypic adhesions, the activation of cells into the cell cycle and the suppression of either spontaneous or induced apoptosis. The latter change in both GC B cells and a group I BL cell line could be engendered in an epitope-unrestricted manner by all eight mAb in soluble form (Table 3) and, as monomeric sCD40L could promote rescue of cells from apoptosis (Fig. 6), with presumably minimal cross-linking of the receptor. For resting B cells, epitope-dependent stimulation of homotypic adhesions via CD40 was apparent (Table 3) while entry into active cell cycle necessitated that the CD40 signal was delivered in an extensively cross-linked form (Figs 4 and 5). A model has been proposed for CD40–CD40L interaction (26) in which CD40 binds to two adjacent CD40L monomeric subunits, suggesting that extensive aggregation of the recep-

Table 3. Ability of CD40 mAb and sCD40L trimer to promote homotypic adhesion and rescue cells from apoptosis

	BL-OGY/C4	EA5	5C3	S2C6	G28-5	HB14	M2	M3	sCD40LT
Homotypic adhesion[a]	±	+	–	++	++++	++	++++	++++	+++
GC B cell rescue[b] (%)	49	69	54	20	51	60	69	49	74
L3055 cell rescue[c] (%)	87	74	77	78	86	80	80	86	80
L3055 cell DNA synthesis[d] (%)	76	70	58	72	85	75	78	80	84

CD40 mAb were added to cultures at 1 μg/ml. sCD40LT was added as a 1/10 dilution of culture supernatant from transfected COS cells.
[a]Homotypic adhesion in cultures of resting tonsillar B cells estimated after 48 h.
[b]Rescue as defined in Methods after 24 h in culture.
[c]As (b) in the presence of anti-μ mAb, BU1 (10 μg/ml).
[d][^{3}H]Thymidine uptake by L3055 cells after 24 h culture in the presence of anti-μ mAb, BU1 (10 μg/ml) expressed as a percentage of the value for cells cultured in the absence of BU1.
Data are means from three experiments except for homotypic adhesion which are representative of three experiments.

Table 4. Ability of CD40 mAb and sCD40L trimer to co-stimulate DNA synthesis in resting B cells.

	BL-OGY/C4	EA5	5C3	S2C6	G28-5	HB14	M2	M3	sCD40LT	CM
CM	3744	4098	3164	4554	5180	2812	4968	5019	4732	422
IL-4	15,943	12,707	14,822	23,160	28,801	18,427	26,944	24,537	32,004	645
PMA	48,034	42,188	39,554	44,632	58,085	47,936	51,230	55,436	50,629	5241

Data are the mean of triplicate counts for [^{3}H]thymidine uptake by 100,000 resting tonsillar B cells after culture for 2 days with CD40 mAb (1 μg/ml) or sCD40LT (1 μg/ml) in the presence of IL-4 (20 ng/ml), PMA (1n M) or CM alone. SEM < 10% of the mean values.

Table 5. Interplay between CD40 mAb and sCD40L trimer in stimulating resting B cells to DNA synthesis

	sCD40LT	BL-OGY/C4	EA5	5C3	G28-5	S2C6	HB14	M2	M3	CM
BL-OGY/C4	**<u>26,232</u>**[a]	2370	3619	2548	2470	4355	3922	2466	2418	2709
EA5	**<u>50,421</u>**	3699	5218	4183	3107	4173	3786	4952	4769	5132
5C3	**<u>28,475</u>**	2807	5038	2206	2649	4772	3622	<u>6400</u>[b]	<u>6580</u>	1991
G28-5	6049	2797	3419	2751	2994	2909	3833	3293	3171	2716
S2C6	**<u>24,318</u>**	4173	3579	4693	2606	5398	2174	4637	4552	4152
HB14	**<u>10,243</u>**	3339	2868	3100	3524	1757	3481	3149	3246	4106
M2	3134	1845	3404	<u>6931</u>	2874	4189	3401	3464	3503	3175
M3	3509	2261	3751	<u>7562</u>	2720	4479	3540	3774	3778	3399
CM	4073	2709	5132	1991	2716	4152	4106	3175	3399	322

Data are the mean of triplicate counts for [^{3}H]thymidine uptake by 100,000 resting tonsillar B cells after culture for 48 h with sCD40LT (1 μg/ml) and CD40 mAb (1 μg/ml) or with combinations of two CD40 mAb. SEM < 10% of the mean values.
[a]Bold and underlined values indicate synergy between agonists.
[b]Underlined values indicate additive effect of agonists.

tor following interaction with soluble trimeric CD40L would be unlikely. The inability of the ligand in this form to stimulate high-rate DNA synthesis in resting cells (Table 4 and Fig. 6) may therefore reflect limited CD40 cross-linking.

It is possible that the differing parameters necessary for influencing resting and GC B cells via CD40 relate more to the phenotypic change engendered rather than a fundamental difference in the populations *per se*. Thus, while the extracellular requirements to rescue GC B cells from apoptosis via CD40 are clearly less demanding than for the promotion of either clustering or cell cycle progression in resting B cells, the ability to sustain GC B cells in active cycle necessitates a similarly high degree of CD40 cross-linking to that noted for the resting population (Fig. 4). This is consistent with our earlier observations that transfectants expressing human CD40L can maintain proliferation of GC B cells, whereas soluble CD40 mAb cannot. Despite this, there does, however, appear to be a distinction between resting and GC B cells in the way that CD40 couples to intracellular signal transduction pathways. In GC B cells, extensive phosphorylation on tyrosine

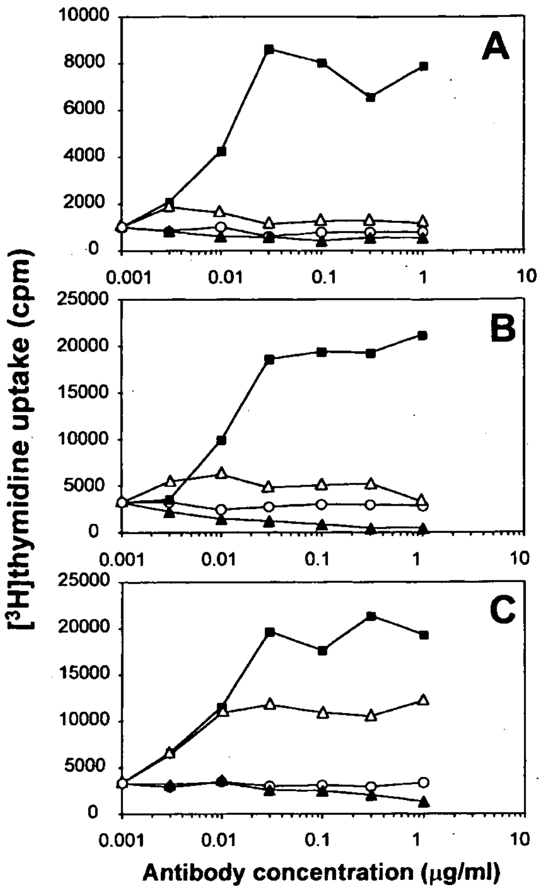

Fig. 3. Epitope-dependent cooperation between CD40 mAb and sCD40L trimer in stimulating DNA synthesis by peripheral blood B cells. Peripheral blood B cells (5×10^4/well) were cultured for 72 h with CD40 mAb and a suboptimal concentration of sCD40LT (0.5 μg/ml) after which [^{3}H]thymidine uptake was determined. (A) Cells cultured without additional co-factors; (B) in the presence of rabbit anti-IgM-Sepharose (5 μg/ml); (C) in the presence of IL-4 (5 ng/ml). ■, EA5; ▲, M2; △, S2C6; O, mouse IgG

residues of multiple substrates has been reported (9) while for resting tonsillar B cells, either no, or limited transient, tyrosine phosphorylation has been described (9,27). It is of course possible that once GC B cells have received their initial protein tyrosine kinase-dependent survival signal via CD40, the receptor recouples to signal transduction pathways operative in the resting B cell to determine exit from, or maintenance of, cell cycle depending on the presence or absence, strength and nature, of subsequent CD40–CD40L interactions. That the requirements for rescuing group I BL cells from induced apoptosis mirrored those of suppressing spontaneous programmed death in GC B cells (Table 3) is compatible with this neoplasm being a phenotypic counterpart to the GC population and suggests that the coupling of CD40 to intracellular signal transduction pathways may be similar in these two populations.

While our studies highlight the differing extracellular requirements for promoting differential phenotypic change, it is interesting to note two recent studies detailing differential signalling through distinct cytoplasmic domains of CD40. Hostager *et al.* (28) demonstrated that a 22 amino acid truncation of residues 236–257 at the C-terminus abrogated or severely impaired the ability of CD40 to signal for increased expression of CD23, B7-1, Fas, LFA-1 and ICAM-1, while leaving B cell receptor (BCR)-dependent enhancement of CD40-stimulated antibody production intact. By contrast, an Ala subtitution of Thr234 left the pathways leading to enhanced expression of LFA-1 and ICAM-1 relatively unscathed but abrogated CD40's capacity to stimulate both the up-regulation of CD23, B7-1 and Fas, and the BCR-dependent enhancement of CD40-stimulated antibody production. It was suggested that the hCD40T^{234}A mutant may be defective through an inability to couple to the TNFR-associated factor-3 (TRAF3), while the defect in the 236–257 C-terminus deletion mutant would result from its inability to recruit TRAF2. Goldstein and Watts (29) reported that both threonine residues 227 and 234 were critical for CD40-dependent B7-1 induction but not for growth inhibition; indeed, a deletion mutant of hCD40 with only six residues remaining in the cytoplasmic tail still retained some capacity to deliver a growth inhibitory signal to transfected murine lymphoma B cells. Although there is no direct evidence at this stage, it is tempting to speculate that the way CD40 is engaged outside the cell may influence the signalling domains activated inside the cell for engendering functional change.

Another possibility that could be considered to explain the apparent differing requirements for signalling GC and resting B cells via CD40 is that the former but not the latter express a CD40L. This could be the already-characterized T cell-associated CD40L (30,31) or a novel B cell-associated counter-structure (15). It is interesting to note that, in all three studies, expression of a CD40L was restricted to activated B cells. If GC B cells—or at least a subset of them—were to express a functional CD40 counter-structure, then this could potentially cooperate with any added CD40 signal to engender phenotypic change.

The finding that mAb binding to distinct epitopes of CD40 can function differently (Tables 3 and 4) demonstrates that there may be an allosteric component to some aspects of CD40 signalling. Although this would be consistent with, it by no means demonstrates, the existence of a second CD40 counter-structure. One possibility to account for the differential behaviour of the CD40 mAb is that they differ in their ability to promote, or inhibit, CD40 microaggregates at the B cell surface. The relative spatial relationships between epitopes defined by the mAb studied and the CD40L binding site appears to be as shown in Fig. 7. This assignment is based on the following information from the present study: (i) competition of CD40L binding (Fig. 2 and Table 2), (ii) competitive binding with CD40 mAb S2C6 (Table 1), and (iii) interplay between mAb with each other and with sCD40LT for promotion of DNA synthesis in resting B cells (Table 5).

CD40 mAb EA5 and 5C3, which could partially inhibit equilibrium binding of sCD40LT to immobilized CD40–Fc (Table 2), apparently also accelerated receptor–ligand exchange under the same conditions (Table 2). The likely

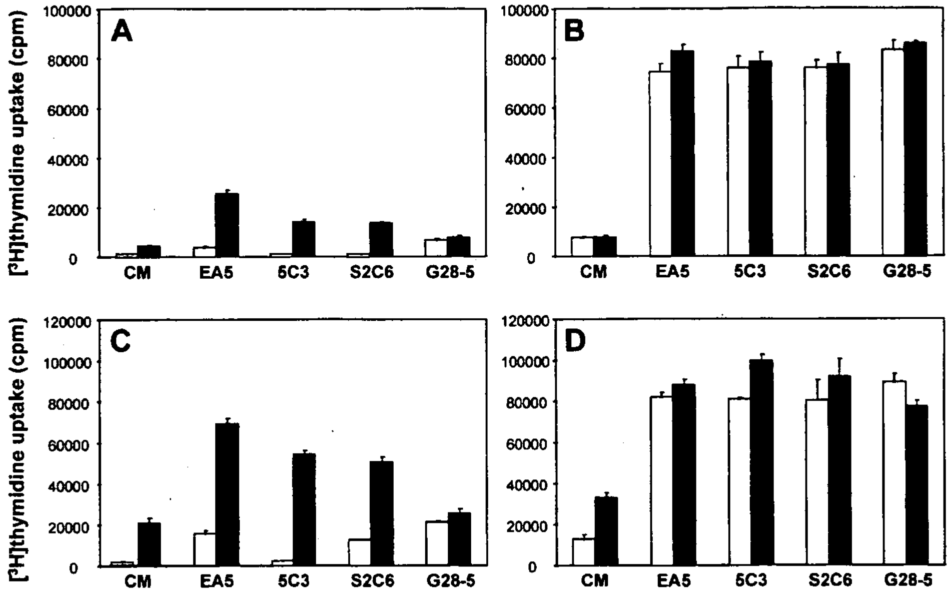

Fig. 4. Stimulation of DNA synthesis in tonsillar B cell subsets by CD40 mAb and sCD40L trimer: influence of co-culture with CD32 transfectants. Tonsillar resting B cells (A and B) or GC B cells (C and D) (100,000 cells/well) were cultured with CD40 mAb (1 μg/ml) in the presence (closed bars) or absence (open bars) of sCD40LT (1/5 dilution of CD40LT-transfected COS cell supernatant). (B and D) Responses of cells cultured with CD32-L cell transfectants (10,000/well). [^{3}H]Thymidine uptake was determined after 48 h. Data are means ± SEM for triplicate measurements. Responses to stimulation with PMA (1 nM) and ionomycin (0.8 μg/ml) were 80,000–90,000 c.p.m. for resting B cells and 90,000–100,000 c.p.m. for GC B cells.

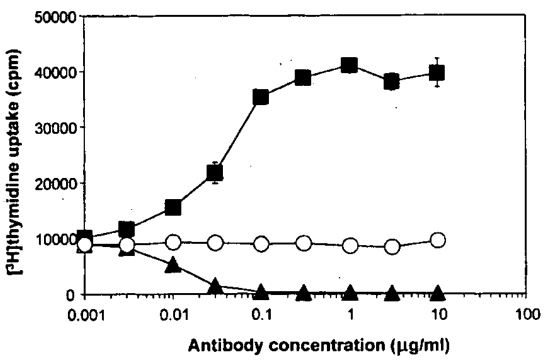

Fig. 5. Stimulation of DNA synthesis in peripheral blood B cells by trimeric sCD40L after cross-linking by antibody. Peripheral blood B cells (5×10^4/well) were cultured for 72 h with sCD40LT (500 ng/ml) in the presence of CD40L mAb M79 (■), M91 (▲) or mouse IgG (O) after which time [^{3}H]thymidine uptake was determined. M91 blocks CD40L binding while M79 does not (18).

mechanism for this is that the ability of the trivalent ligand to stably cross-link this bivalent form of the receptor is altered when these mAb are bound. Enhanced binding of CD40–Fc to the CD40L expressed on T cells after pre-incubation with 5C3 (Table 1) may reflect the ability of the antibody to promote further cross-linking of receptor–ligand complexes at the cell surface by interacting with epitopes outside the ligand binding site.

In an earlier study, Bjork *et al.* (32) also found that antibody S2C6 was able to partially block CD40L binding and noted that—in the presence of IL-4—S2C6 showed cooperation with sCD40LT for stimulating both DNA synthesis and IgE production in human B cells. Using a different set of mAb to the ones investigated in detail in our study, Lindhout *et al.* (33) found that whilst they were poor at stimulating resting B cells into proliferation, they were capable of suppressing apoptosis in the 'light density' B cell fraction from tonsils: these would have included GC B cells and is thus consistent with the notion that there are epitope differences in terms of the function engendered through CD40. Probably due to the fewer number of antibodies to CD40 available, there is less information on epitope distribution and function in mouse but Heath *et al.* (14) have reported on two mAb which appear to bind to different sites on murine CD40 and exhibit differential behaviour.

Teleologically, it is not readily apparent as to why the requirements for rescuing GC B cells via CD40 should be substantially less rigorous than for stimulating B cells into and through cycle. It may possibly relate to the relatively low (but not insignificant) number of T cells available at the presumed physiological site of CD40-dependent rescue of antigen-selected centrocytes in the GC light zone (34) such that if the demands were too high then useful V gene mutations could be lost through insufficient signalling. This would need

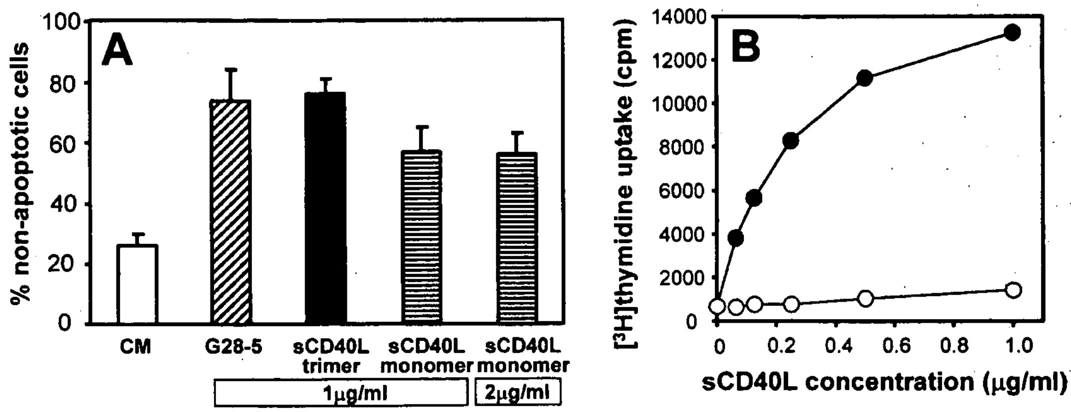

Fig. 6. Monomeric sCD40L can rescue GC B cells from apoptosis but cannot stimulate resting B cells into cell cycle. (A) Tonsillar GC B cells (10^6/ml) were cultured with CM alone, CD40 mAb G28-5 (1 μg/ml), sCD40L trimer (1 μg/ml) or sCD40L monomer (1 or 2 μg/ml). The percentage of non-apoptotic cells remaining was determined after 24 h. Data are means ± SD for three experiments. (B) Tonsillar resting B cells (100,000/well) were cultured with sCD40L trimer (●) or monomer (O) at various concentrations and [^{3}H]thymidine uptake was determined after 48 h.

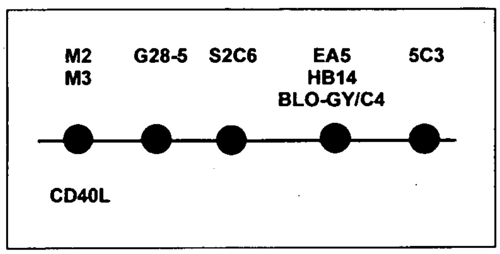

Fig. 7. Spatial relationship between epitopes on CD40 suggested by this study.

to be balanced with the possibility that any naturally produced soluble CD40L in the GC could rescue worthless mutations in a non-cognate fashion. Perhaps the rare escape of an autoreactive mutation as evidenced in rheumatoid arthritis or systemic lupus erythematosus might be accounted for by occasional noise in such fine tuning. It is of interest that continued proliferation—and, thus, possibly the rerouting of centrocytes back to the dark zone to accumulate further mutations in antigen receptor—requires prolonged exposure to cell bound CD40L, suggesting a mechanism for minimizing the production of particularly high-affinity somatic mutations to self.

Soluble CD40L [synonym, TNF-related activation protein (TRAP)] has been reported to be released by activated T_h cells *in vitro* (35) indicating at least the possibility of the above scenario arising *in vivo*. This material, characterized as an 18 kDa monomer, did not result from cleavage of cell surface CD40L but may have been produced by processing within an intracellular compartment (35). Naturally produced soluble CD40L has subsequently been shown to down-regulate CD40 on dendritic cells and to produce a long-lasting anti-apoptotic effect (36). While the monomeric recombinant CD40L used in our study was able to rescue GC B cells from apoptosis we wish to stress that this does not necessarily imply that univalent tethering of CD40 is sufficient for such rescue. Although monomeric at the concentration maintained in solution, it is possible that once bound to the cell surface a degree of spontaneous aggregation occurs sufficient to engender a functional signal. The only conclusion we can, or wish to, reach is that the experiments using 'monomeric' CD40L further highlight that the requirements for suppressing apoptosis and inducing proliferation via CD40 are less rigid for the former than the latter. We are currently exploring the intracellular signal transduction mechanisms that may underlie not only this but also the differences observed in CD40 mAb to promote homotypic adhesions.

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Abbreviations

AET	aminoethylisothiouronium bromide
BCR	B cell (antigen) receptor
BL	Burkitt's lymphoma
CD40L	CD40 ligand
CM	culture medium
GaHIg	goat anti-human IgG1
GC	germinal center
NGFR	nerve growth factor receptor
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PMA	phorbol myristate acetate
sCD40L	soluble CD40 ligand
sCD40LT	soluble CD40 ligand trimer
SPR	surface plasmon resonance
SRBC	sheep red blood cell
TNFR	tumour necrosis factor receptor
TRAF	TNFR-associated factor

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Technical Data Sheet

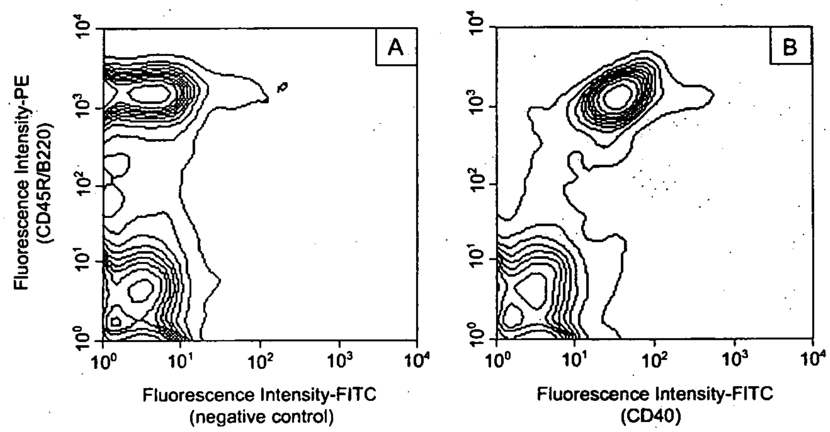
Purified Hamster Anti-Mouse CD40 Monoclonal Antibody

Product Information

Catalog Number:	**553722**
Size:	0.1 mg
Clone:	HM40-3
Immunogen:	(BALB/c x NZB)F_1 mouse-derived lymphoma WEHI-231[1,2]
Isotype:	Armenian Hamster IgM, κ
Storage Buffer:	Aqueous buffered solution containing 0.09% Sodium Azide.

Specificity

The HM40-3 antibody reacts with CD40,[1,2,3] a 40-50–kDa glycoprotein expressed on B lymphocytes and other antigen-presenting cells.[4,5,6,7] The CD40 molecule has a central role in B-cell growth and differentiation.[5,8,9] Furthermore, interactions of CD40 with its ligand, CD154, are involved in the initiation and effector stages of cell-mediated immune responses.[5] CD40 may be involved in the triggering of NK cells[10] and NK-T cells.[11] Soluble HM40-3 antibody stimulates splenic and peritoneal B cells to proliferate *in vitro*.[2,3,12] This antibody also induces spleen B cells to express the costimulatory molecules CD80 (B7-1) and CD86 (B7-2).[2] HM40-3 mAb has been demonstrated to inhibit the binding of soluble CD154 (gp39, CD40 Ligand) to soluble CD40 and to cell-surface CD40.[13] This hamster mAb to a mouse leukocyte antigen has been observed to cross-react with similar populations of Lewis,[14,15] Sprague-Dawley,[15] and LOU[16] rat leukocytes.



Two-color analysis of the expression of CD40 on mouse spleen cells. BALB/c splenocytes were simultaneously stained with PE-conjugated anti-mouse CD45R/B220 mAb RA3-6B2 (Cat. no. 553089/553090; both panels) and purified HM40-3 mAb (Panel B), followed by FITC-conjugated anti-hamster IgM mAb G188-9 (Cat. no. 554036; both panels). Flow cytometry was performed on a BD FACScan™ flow cytometry system.

Preparation and Storage

The antibody was purified from tissue culture supernatant by affinity chromatography. The antibody solution should be stored undiluted at 4°C.

Usage

This antibody has been tested by immunofluorescent staining (≤ 1 µg/million cells) with flow cytometric analysis to assure specificity and reactivity. Other reported applications include blocking of ligand binding,[13] blocking of NK T-cell activation,[13] and stimulation of mouse B lymphocytes,[2,3,12,17] dendritic cells,[18] and Lewis rat B cells.[14] For immunohistochemical staining of mouse tissue, we recommend the use of purified anti-mouse CD40 mAb 3/23 in our special formulation for immunohistochemistry, Cat. no. 550285. **Since applications vary, each investigator must determine dilutions appropriate for individual use.**

Caution: Sodium azide is a reversible inhibitor of oxidative metabolism; therefore, antibody preparations containing this preservative agent must not be used in cell cultures nor injected into animals. Sodium azide may be removed by washing stained cells or plate-bound antibody or dialyzing soluble antibody in sodium azide-free buffer. Since endotoxin may also affect the results of functional studies, we recommend the NA/LE™ (No Azide/Low Endotoxin) antibody format for *in vitro* and *in vivo* use.

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***Hazardous Ingredient: Sodium Azide.** Avoid exposure to skin and eyes, ingestion, and contact with heat, acids, and metals. Wash exposed skin with soap and water. Flush eyes with water. Dilute with running water before discharge into plumbing.*

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EXHIBIT C

BD Pharmingen™

Technical Data Sheet

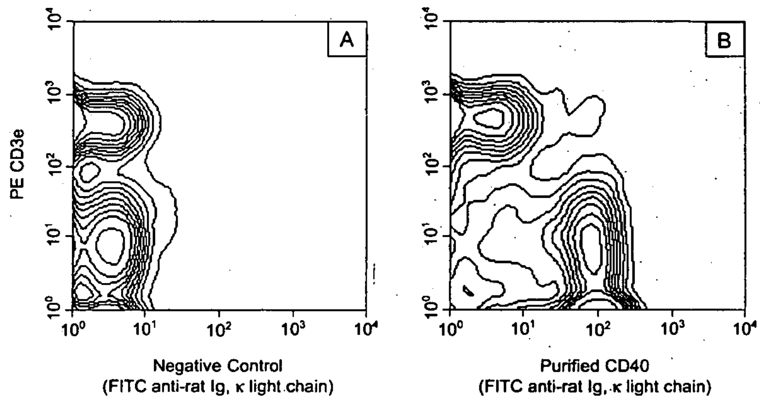
Purified Rat Anti-Mouse CD40 Monoclonal Antibody

Product Information

Catalog Number:	**553788**
Size:	0.5 mg
Clone:	3/23
Immunogen:	Recombinant mouse CD40[1]
Isotype:	Rat (Lou) IgG_{2a}, κ
Storage Buffer:	Aqueous buffered solution containing 0.09% Sodium Azide.

Specificity

The 3/23 antibody reacts with CD40, a 40-50–kDa glycoprotein expressed on B lymphocytes and other antigen-presenting cells.[1,2,3,4,5,6] In some mouse strains, the 3/23 mAb also reacts with 5-10% of T lymphocytes in the adult, but not neonatal, mouse spleen,[1,7] and CD40 has been reported to be transiently expressed on activated $CD4^+$ and $CD8^+$ T cells.[8] The CD40 molecule has a central role in B-cell growth and differentiation.[2,4,9,10] Furthermore, interactions of CD40 with its ligand, CD154, are involved in the initiation, effector, and memory stages of cell-mediated immune responses.[4,8] CD40 may be involved in the triggering of NK cells[11] and NK-T cells.[12] Ligation of CD40 with the 3/23 antibody can induce splenic B cells to express the costimulatory molecule CD86 (B7-2).[1] In addition, although 3/23 antibody by itself is a weak B-cell mitogen, it synergizes markedly with mitogenic anti-IgM or anti-IgD mAb or IL-4 to promote B-cell proliferation.[1,13]



Two-color analysis of the expression of CD40 on mouse spleen cells. BALB/c splenocytes were simultaneously stained with PE-conjugated anti-mouse CD3e mAb 145-2C11 (Cat. no. 553063/553064) and purified mAb 3/23 (Panel B), followed by FITC-conjugated anti-rat Ig, κ light chain mAb MRK-1 (Cat. no. 553872). Please note that staining of a T-cell subset by mAb 3/23 has not been consistently observed. Flow cytometry was performed on a BD FACScan™ flow cytometry system.

Preparation and Storage

The antibody was purified from tissue culture supernatant by affinity chromatography. The antibody solution should be stored undiluted at 4°C.

Usage

This antibody has been tested by immunofluorescent staining (≤ 1 μg/million cells) with flow cytometric analysis to assure specificity and reactivity. Other reported applications include *in vitro* B-cell activation studies,[1,13] enhancement of *in vivo* anti-tumor response,[14] and immunohistochemical staining (IHC) of acetone-fixed frozen sections,[6] but not formalin-fixed paraffin-embedded sections.[15] For IHC, we recommend the use of purified 3/23 mAb in our special formulation for immunohistochemistry, Cat. no. 550285. **Since applications vary, each investigator must determine dilutions appropriate for individual use.**

Caution: Sodium azide is a reversible inhibitor of oxidative metabolism; therefore, antibody preparations containing this preservative agent must not be used in cell cultures nor injected into animals. Sodium azide may be removed by washing stained cells or plate-bound antibody or dialyzing soluble antibody in sodium azide-free buffer. Since endotoxin may also affect the results of functional studies, we recommend the NA/LE™ (No Azide/Low Endotoxin) antibody format for *in vitro* and *in vivo* use.

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